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Abnormal Development of Preimplantation Embryos Derived from Intersubspecific Hybrids between *Mus musculus molossinus* and *M. m. domesticus*

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ABSTRACT—Japanese wild mice, *Mus musculus molossinus,* are genetically remote from laboratry mice which are derived predominantry from European wild mice *M. m. domesticus.* Our previous study demonstrated that F2 progeny between these two mouse subspecies show low ferility, even though F1 hybrids are fully fertile. In fact about half of the F2 females fail to become pregnant. We examined the *in vitro* development of preimplantation embryos from F2 progeny between MOM (one of the inbred strains derived from Japanese wild mice) and C57BL/6 (B6, an inbred strain of laboratory mice). We found that the low pregnancy rate of F2 females results from a high embryonic mortality: only 58.4% (66/113) of embryos developed to the blastocyst stage in the F2×F2 cross, whereas in the B6×B6 cross the corresponding figure was 100.0% (23/23). The mortality was not due to defects in sperm from F2 males but rather to defects in the eggs from F2 females: the survival rate of embryos up to the blastocyst stage was 52.2% (82/157) in the F2♀×B6♂ cross, whereas it was 94.9% (74/78) in the B6♀×F2♂ cross. The factors responsible for this mortality are attributable to nucleus, not to maternally inherited cytoplasm: more than 10% of N2 femals derived from backcrossing either F1 femals to B6 males or F1 males to B6 females showed the higher embryonic mortality. This finding suggests that intersubspecies genic combinations, either intragenic or intergenic, give rise to some deleterious effects on the oocytes during oogenesis.

INTRODUCTION

The species *Mus musculus* can be classified into several groups (subspecies) from a genetic perspective [1, 2]. It has been demonstrated that common laboratory mice originate predominantly from a European subspecies, *Mus musculus domesticus,* by the analysis of mitochondrial and nuclear genomes [3, 4]. Japanese wild mice, *M. m. molossinus,* are very different from *M. m. domesticus* and are closely related to *M. m. musculus* and M. m. castaneus [2, 3]. The evolutionary divergence of *M. m. molossinus* from *M. m. domesticus* is estimated to have oocurred about one million years ago [3, 5, 6].

There appears to exist a severe restriction of the gene flow in the contact zone between *M. m. domesticus* and *M. m. musculus* in Europe [7]. It can be inferred, therefore, that there is an incompatibility between the genomes of the two subspecies. Male sterility is suspected of being one manifestation of the incompatibility that is responsible for restriction of the gene flow in this area [7, 8].

Several inbred strains derived from Japanese wild mice, *Mus musculus molossinus,* have been established in our laboratory to provide us with a wide variety of experimental material. When we attempted to generate recombinant inbred strains from F1 hybrids between molossinus and laboratory strains, a serious depression in fertility was observed in the subsequent generations, despite the fact that both parental strains had been bred for more than 20 generations by brother-sister matings. So far, no evidence has been reported that there is a serious reproductive disturbance in the F2 females derived from inter-strain crosses among laboratory strains of inbred mice. We previously reported details of the reproductive

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preformance of F1 and F2 generations from crosses between molossinus and laboratory strains [9]. Such F1 hybrids are fully fertile, but in the F2 generation, about half of the F2 females fail to become pregnat. Since the F2 progeny copulated normally, it appeared possible that preimplantation loss of embryos may be responsible for the infertility observed in half of the F2 females.

In the present study, we examined the development *in vitro* of preimplantation embryos obtained from F2 and backross (N2) generations between molossinus and loboratory mice.

MATERIALS AND METHODS

Animals

C57BL/6 (B6) and MOM strains were used. The MOM strain is derived from Japanese wild mice (*Mus musculus molossinus*), whose ancestors were captured at Nagoya in Japan. The number of inbreeding generatons was 46 at the time when the present study was undertaken. F1 and F2 generations were produced through reciprocal matings between B6 and MOM. Since there were no differences in the fertility between members of the F2 generations derived from (B6♀×MOM♂) F1 and from (MOM♀×B6♂) F1, they were pooled in the present study. Backcross progeny (N2) were produced from the crossing of either F1♀×B6♂ or B6♀×F1♂.

Observation of embryos

Two- to twelve-month-old females were mated with males and checked daily for vaginal plugs. The day on which a plug was found to be present was designated as Day 0 of pregnancy. The embryos were recovered by flushing oviducts with Medium 2 (M2) [10] from plug-positive females on Day 1 or Day 2, and they were examined with reference to developmental stage and morphology under a dissecting microscope. Subsequently, the embryos were cultured in pre-equilibrated Medium 16 (M16) [11] under paraffin oil in an atmosphere of 5% CO_2 in air at 37°C. Embryos were examined at intervals of 24 hr and the number of embryos that developed to the blastocyst stage was recorded.

RESULTS

Our of a total of 21 embryos obtained from three MOM females mated with MOM males, 18 (85.7%) developed into expanded blastocysts. Most embryos from F1 females which had been mated with B6, MOM, or F1 males also developed to the blastocyst stage: out of 103 morphologically normal embryos collected from 13 females, 95 (92.2%) developed into blastocysts. In these crosses, embryos were obtained on Day 1 and Day 2. As summarized in Table 1, ovulation and feritilization occurred normally in the F2 females.

All fertilized eggs were cultured *in vitro* and the number of embryos that developed to the blasto-

TABLE 1. Developmental ability of embryos obtained on Day 1 of pregnancy from F2 females between B6 and MOM strains and B6 females

Crosses ♀×♂	No. of ♀♀ examined	No. of eggs collected (mean±sem)	No. of normal embryos[a]	No. of embryos that developed to blastocysts during 4 days in culture
F2×F2	16	114 (71.±0.5)	113 (99.1%)	66 (58.4)[b]
F2×B6	24	166 (6.9±0.4)	157 (94.6)	82 (52.2)
B6×F2	9	80 (8.9±0.5)	78 (97.5)	74 (94.9)
B6×B6	3	23 (7.7±0.3)	23 (100.0)	23 (100.0)

[a] Morphologically normal 2- to 6-cell embryos were counted.
[b] Percentage was computed from the number of normal embryos.

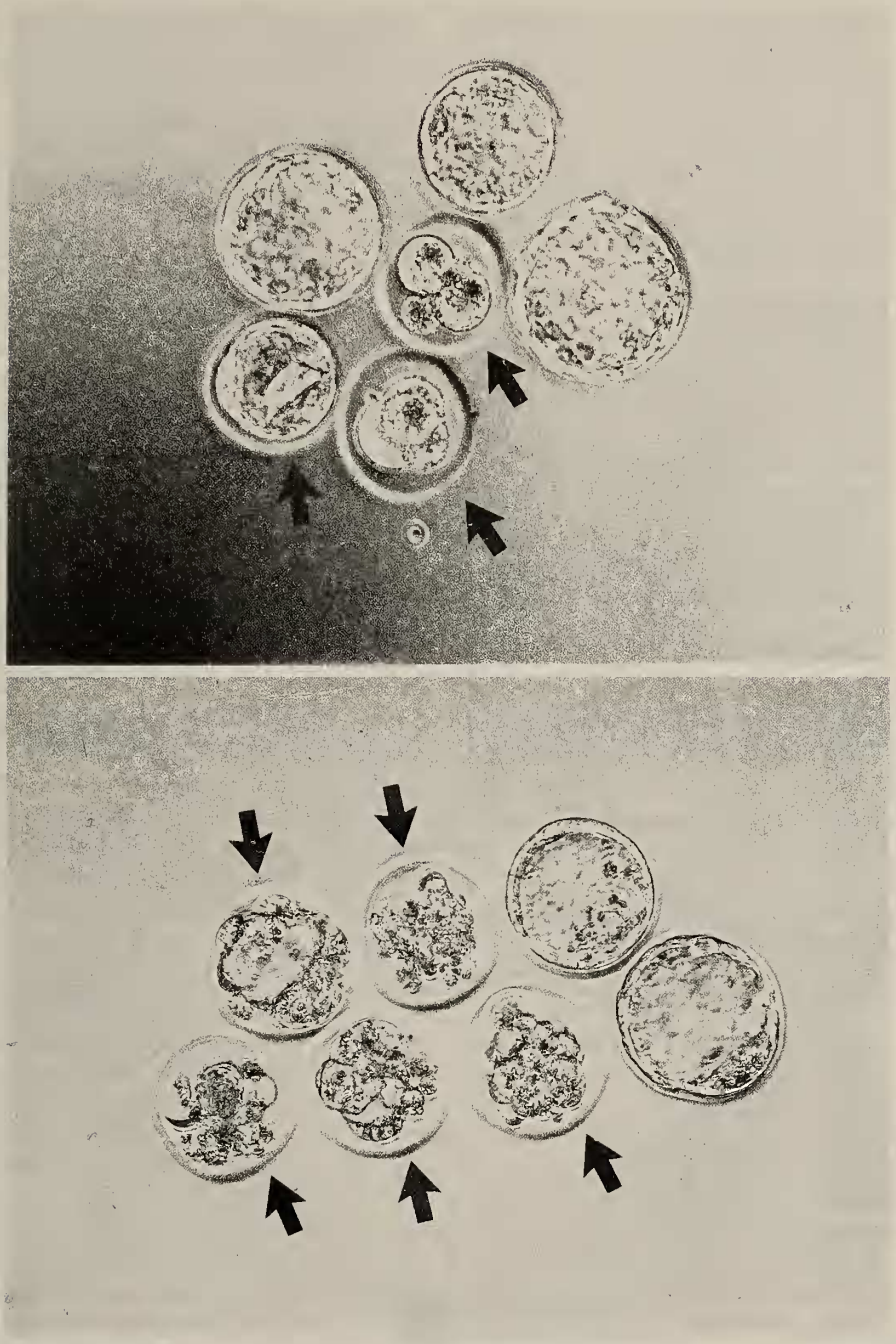

FIG. 1. Morphological appearance after culture *in vitro* of embryos from F2 ♀ × B6 ♂ cross, including degenerated or retarded embryos. Embryos were collected on Day 1 of pregnancy and photographed after 72 hr in culture. Arrows indicate the abnormal embryos that are developmentally retarded or have degenerated.

TABLE 2. Proportion of normal embryos on Day 2 of pregnancy from F2 and B6 females mated with B6 males, and their developmental ability in culture

Crosses ♀×♂	No. of ♀♀ examined	No. of embryos obtained[a)]	No. of normal embryos[a)]	No. of embryos that developed to blastocysts during 3 days in culture
F2×B6	22	161	118 (73.3%)	95 (59.0%[c)])
B6×B6	7	53	53 (100.0)	50 (94.3)

[a)] Embryos, and not unfertilized eggs, were counted.
[b)] Morphologically normal embryos at the 6-cell to the morula stage were counted.
[c)] No. of blastocysts/No. of embryos obtained.

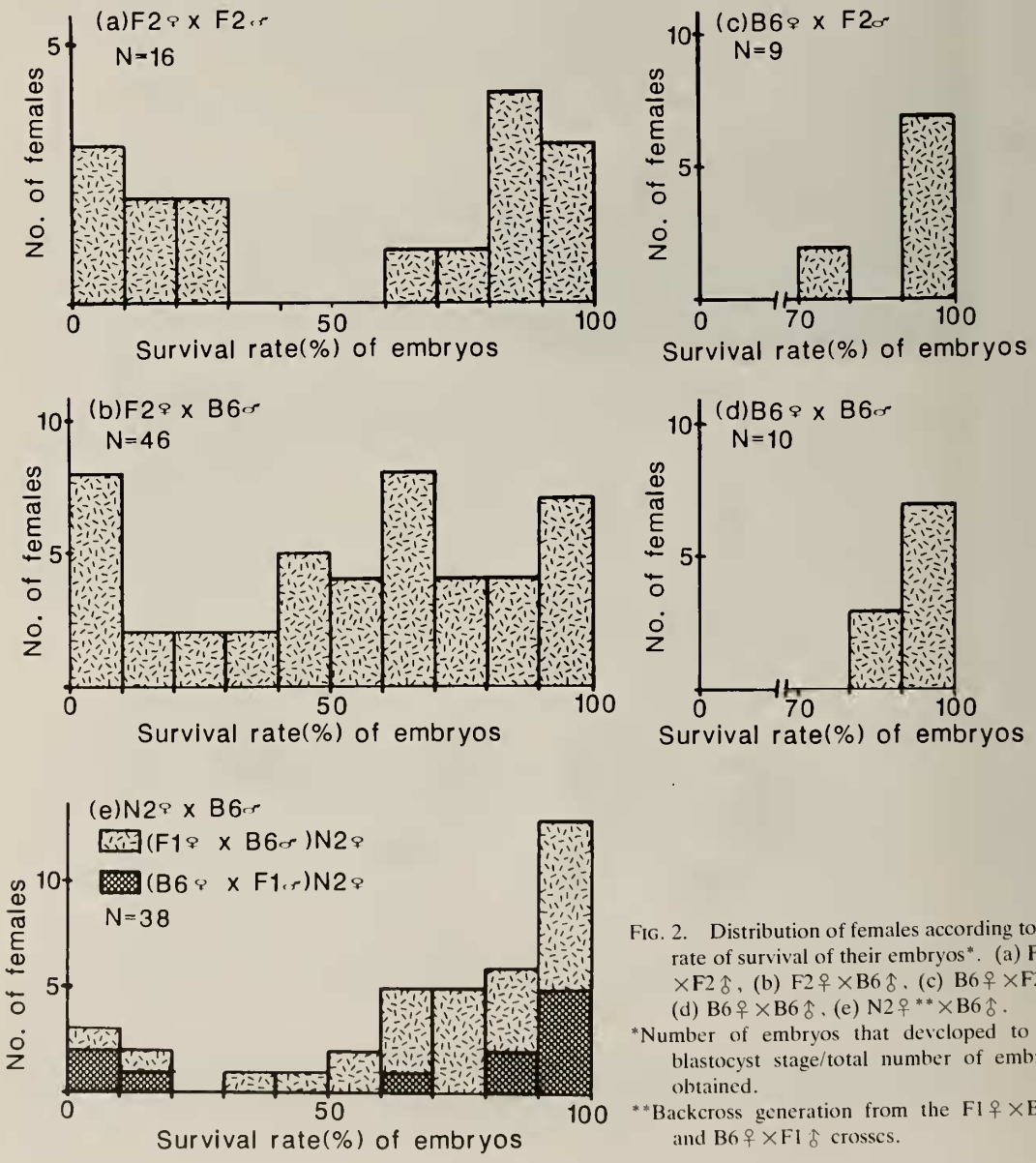

FIG. 2. Distribution of females according to the rate of survival of their embryos*. (a) F2♀×F2♂, (b) F2♀×B6♂, (c) B6♀×F2♂, (d) B6♀×B6♂, (e) N2♀**×B6♂.

*Number of embryos that devcloped to the blastocyst stage/total number of embryos obtained.

**Backcross generation from the F1♀×B6♂ and B6♀×F1♂ crosses.

cyst stage was counted. In the crosses of F2×F2 and F2♀×B6♂, the proportion of embryos that developed to the blastocyst stage, including unexpanded embryos, was very small (Table 1). Many embryos from F2 females exhibited morphological alterations or developmental arrest at various stages from the 2- to the 6-cell stage as far as blastulation. Figure 1 shows embryos from F2 females during culture. They were all morphologically normal, 2- to 4-cell embryos when collected on Day 1. In the case of the B6♀×F2♂ and B6× B6 crosses, almost all embryos developed into normal, expanded blastocysts. These results suggest that the abnormality in development can be attributed to some disturbance in the eggs from F2 females.

Next we examined the embryos on Day 2 of pregnancy from F2 and B6 frmales mated with B6 males. As shown in Table 2, more than 70% of embryos collected from F2♀×B6♂ crosses were morphologically normal at the 6-cell to the morula stage while the others were abnormal or had degenerated. During culture, about 60% of embryos survived to form blastocysts. This result confirmed the findings shown in Table 1: about half of the embryos from F2 females died around the morula stage both *in vivo* and *in vitro*. In the control cross, B6×B6, morphologically normal embryos were obtained and most of them developed to form expanded blastocysts.

Figure 2 shows the distribution of females according to the proportion of embryos that developed to the blastocyst stage. The results of F2♀×F2♂ and F2♀×B6♂ crosses (Fig. 2a, b) demonstrated that there are two types of female: one showing a higher rate of survival of embryos and the other showing a lower rate. In the crosses of B6♀×F2♂ and B6♀×B6♂, the rate of survival of embryos that developed to the blastocyst stage for each female was greater than 70% (Fig. 2c, d). This result demonstrates that the F2 sperm does not negatively influence the developmental abnormality of the embryos. Figure 2e shows the distribution of the survival rates of embryos obtained from N2 females, progeny of the F1♀×B6♀ and B6♀×F1♂ crosses, when they were mated with B6 males. The mean number of embryos obtained on Day 2 was 8.2±0.3. Extremely poor rates of survival of embryos, lower than 20%, were observed in the case of 5 N2 females out of 38 (13.2%); 3 of these females being from the B6♀×F1♂ cross, and 2 from the F1♀× B6♂ cross. The higher rates of embryonic mortality observed in the case of these N2 females demonstrate that the abnormality is inherited from both F1 females and F1 males.

DISCUSSION

In order to investigate the infertility of about half of the F2 females between the two subspecies *Mus musculus domesticus* and *M. m. molossinus*, we examined the preimplantation development of their embryos. Our data indicate that a higher frequency of embryonic mortality during preimplantation development is responsible for the infertility observed in the F2 females. We confirmed our previous observation that there is segregation of fertile and infertile F2 females. Some F2 females showed higher rates of survival of their embryos and others showed lower rates. This embryonic death can be attributed to defects in the eggs, not in the sperm, from the F2 generation. F2 males are fertile when they mate with B6 females [9]. The factors that cause the embryonic death which occurs around the morula stage are inherited from both F1 males and F1 females (Fig. 2e). It has been reported that embryonic genes are transcribed and expressed soon after fertilization [12, 13]. Such a conclusion implies the possible switching of the information for development from maternal to embryonic [12]. The early expression of embryonic genes is necessary for compaction of 8-cell mouse embryos [13]. Therefore, the mortality at the early developmental stage observed in our study may possibly be due to some abnormality in the embryonic genome and not in the maternally inherited cytoplasm which functions in the early stages of development. In addition, the factors that cause this abnormality appear to be autosomal. Some of the N2 females that showed lower rates of suvival of embryos were derived from the B6♀×(B6♀×MOM♂)F1♂ cross, having two B6 X-chromosomes.

The reproductive abnormality in the F2 generation observed in this study is presumably related to

a genetic incompatibility that arises from the combination of genes (chromosomes) from different subspecies. Such an incompatibility is observed in the contact zone between *Mus musculus domesticus* and *M. m. musculus* in Europe, where a restriction of the gene flow is observed [7, 14, 15]. Hybrid male sterility has been considered to be responsible for this restriction. Recently Vanlerberghe *et al.* [15] reported that the limitation of Y-chromosome introgression in the contact zone is not attributable to the sterility in F1 males between the two subspecies. The developmental abnormalities in the preimplantation embryos from F2 females of the intersubspecific crosses observed in this study provide one possible explanation. From an analysis of restriction patterns of mitochondrial DNA, it has been shown that *M. m. molossinus* is related to *M. m. musculus* and to *M. m. castaneus,* both of which are quite remote from *M. m. domesticus* [2, 4, 5, 16]. It has been proposed that *M. m. molossinus* originated from the hybridization of *M. m. musculus* and *M. m. castaneus* [17]. If this was indeed the case, it can be inferred that hybrids between *domesticus* and *musculus* in the contact zone in Europe could show reproductive defects in the same way as the intersubspecific hybrids described in this report. At investigation of this interpretation should prove interesting.

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